



Effect of saliva composition on experimental root caries

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Abstract: The aim of this study was to determine the effect of saliva composition on caries lesion development independently of the flow rate of unstimulated whole saliva (UWS) and other caries-related variables such as lesion progression time, oral hygiene level, and fluoride exposure. We hypothesized that this could be done by developing experimental root caries under carefully controlled conditions in situ in test subjects with UWS flow rates within a narrow window of normalcy. Fifteen female and 5 male subjects (66 +/- 6 years) were selected for the study according to their UWS flow rates between 0.2 and 0.4 ml/min. All subjects developed experimental root caries lesions during a 62-day period in which UWS as well as stimulated whole saliva (SWS) were repeatedly collected and analysed for flow rate, pH, buffer capacity, inorganic, and organic composition. Caries lesion development was determined by quantitative microradiography. The mean UWS flow rate was 0.30 +/- 0.05 ml/min. Significant negative correlations were obtained between UWS total phosphate concentration and mineral loss (DeltaZ; $r(s) = -0.72$, $p < 0.001$) and UWS total protein concentration and DeltaZ ($r(s) = -0.70$, $p < 0.01$). SWS and its constituents had only limited or no effect on DeltaZ. Qualitative UWS protein analysis (SDS-PAGE) revealed that subjects with low DeltaZ values had broader and more stained amylase bands than subjects with high DeltaZ values. These findings were confirmed quantitatively by HPLC. We conclude that, within a group of subjects with normal UWS flow rates, the UWS composition was more important for caries lesion development than the SWS composition. Furthermore, high UWS concentrations of phosphate, protein, and amylase were caries-protective.

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Effect of Saliva Composition on Experimental Root Caries

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Key Words

Amylase · Buffer capacity · In situ caries model · Protein profile · Root caries

Abstract

The aim of this study was to determine the effect of saliva composition on caries lesion development independently of the flow rate of unstimulated whole saliva (UWS) and other caries-related variables such as lesion progression time, oral hygiene level, and fluoride exposure. We hypothesized that this could be done by developing experimental root caries under carefully controlled conditions in situ in test subjects with UWS flow rates within a narrow window of normalcy. Fifteen female and 5 male subjects (66 ± 6 years) were selected for the study according to their UWS flow rates between 0.2 and 0.4 ml/min. All subjects developed experimental root caries lesions during a 62-day period in which UWS as well as stimulated whole saliva (SWS) were repeatedly collected and analysed for flow rate, pH, buffer capacity, inorganic, and organic composition. Caries lesion development was determined by quantitative micro-radiography. The mean UWS flow rate was 0.30 ± 0.05 ml/min. Significant negative correlations were ob-

tained between UWS total phosphate concentration and mineral loss (ΔZ ; $r_s = -0.72$, $p < 0.001$) and UWS total protein concentration and ΔZ ($r_s = -0.70$, $p < 0.01$). SWS and its constituents had only limited or no effect on ΔZ . Qualitative UWS protein analysis (SDS-PAGE) revealed that subjects with low ΔZ values had broader and more stained amylase bands than subjects with high ΔZ values. These findings were confirmed quantitatively by HPLC. We conclude that, within a group of subjects with normal UWS flow rates, the UWS composition was more important for caries lesion development than the SWS composition. Furthermore, high UWS concentrations of phosphate, protein, and amylase were caries-protective.

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It has been shown that individuals who suffer from low salivary flow rates have higher caries experience than individuals with normal salivary flow rates [Rundegren et al., 1985; Papas et al., 1993]. From a theoretical point of view, it has been suggested that the unstimulated saliva flow rate may be more important for this relation than the stimulated one [Dawes, 1983]. The caries-controlling effect of the salivary flow rate can probably be attributed to the rinsing and clearing effect of saliva in the oral

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cavity [Lagerlöf and Dawes, 1985; Bardow et al., 2003]. Apart from these effects human saliva also contains a variety of constituents such as bicarbonate, phosphate, calcium, and proteins that could influence caries lesion development [for reviews, see Lenander-Lumikari and Loimaranta, 2000; Lendenmann et al., 2000; Amerongen and Veerman, 2002]. However, in most clinical studies it has not been possible to demonstrate an effect of saliva composition on caries lesion development. We have previously speculated that it may be difficult to separate the effect of saliva composition from the effect of salivary flow rate on caries lesion progression as saliva composition is a consequence of saliva flow rate [Bardow et al., 2003]. Furthermore, behavioural factors such as oral hygiene, fluoride exposure, and sugar consumption may mask a possible relation between saliva composition and caries development in cross-sectional study designs. Thus, the influence of saliva composition on caries lesion development should be studied in a longitudinal setup under carefully controlled conditions where these variables are either stratified or closely monitored. In situ caries models allow for such conditions including stratification of time, oral hygiene level and fluoride exposure. Furthermore the pattern of daily sugar consumption can be monitored over the experimental period. By including only subjects with unstimulated whole saliva flow rates within a narrow window of normalcy we aimed to determine the effect of saliva composition on the development of experimental root caries in situ independently of saliva flow rate.

Material and Methods

Study Group

The inclusion criteria were that subjects should have an unstimulated whole saliva flow rate in the range between 0.2 and 0.4 ml/min. Within this range caries lesion development has been shown to be independent of the salivary flow rate [Bardow et al., 2001]. Furthermore the subjects should wear a removable partial denture in the lower jaw, be mentally and physically healthy, and capable of participating in the study. Twenty subjects, 15 females and 5 males (mean age 66 ± 6 years) recruited among patients at the Copenhagen Dental School, fulfilled the criteria and were included. Ten subjects were on daily medication, mainly hormone replacement therapy, and 1 subject reported to have dry mouth symptoms occasionally. The subject reporting dry mouth symptoms was also the subject who had the lowest unstimulated saliva flow rate in this study (0.22 ml/min). The Ethical Committee of Copenhagen approved the study (03-005/97).

Study Design

All subjects attended four visits during the study period that lasted 62 days. All visits occurred between 10 a.m. and 2 p.m. At the

outset of the study (day 0), the unstimulated whole saliva flow rate was determined for means of inclusion/exclusion in the project. If the unstimulated saliva flow rate was within the range selected for the study the examination continued and the subjects were interviewed to elicit information about medical history, medication intake, and subjective feeling of oral dryness [Beck et al., 1961]. Sugar intake was scored according to the frequency of intake of the ten main sources for sugar intake distributed on specific foodstuffs on the Scandinavian market [Swedish Nutrition Council, 2002]. The scores for the intake of each of the ten foodstuffs were defined as: never, once a week or less, 2–6 times a week, once or twice a day, and more than twice a day. Finally, a root surface specimen was attached to a recession in the buccal flange of the premolar or molar region of the lower partial denture as previously described [Nyvad et al., 1997]. During the experimental period, the subjects were asked to wear their dentures day and night and to avoid touching or cleaning the specimen. For cleaning their dentures and natural dentition the subjects were supplied with fluoride-free toothpaste (Ren-I-Mund™) by the investigators.

At the second visit (day 14) recording of the plaque index [Silness and Løe, 1964], the gingival index [Løe and Silness, 1963], and the calculus index [Ramfjord, 1967] was performed for all teeth in the lower jaw. Active and inactive enamel and root caries were scored according to Nyvad et al. [1999], and the individual components of DMFS as well as total DMFS were calculated. Finally the unstimulated and paraffin-stimulated saliva flow rates were determined and the collected saliva stored at -80°C for later analyses.

At the third visit (day 45) unstimulated and paraffin-stimulated whole saliva were collected under paraffin oil in a special receptacle without loss of CO_2 for determination of salivary flow rates, pH, buffer capacity, and bicarbonate concentration. These determinations were carried out as described by Bardow et al. [2000a, b] and the remaining saliva was stored at -80°C for later analyses. Finally, a digital close-up picture was taken of the tooth specimen for visual determination of plaque extension.

At the fourth visit (day 62) paraffin-stimulated whole saliva was collected for 5 min for determination of the whole stimulated saliva flow rate and the salivary lactobacilli level by use of a commercial kit (Dentocult LB™). Hereafter the root surface specimen was removed from the denture and fixed [Karnovsky, 1965]. Compliance was evaluated at this and the two preceding visits as previously described [Bardow et al., 2003]. Unless otherwise stated salivary flow rates were determined by the draining method [Navazesh, 1993], chewing stimulation paced by a metronome (55 chews/min), and the flow rates calculated in ml/min (days 0, 14, and 62).

Evaluation of Plaque Extension on the Specimens

Five dentists who were blinded with regard to the study scored visual plaque extension on the tooth specimens from the close-up pictures taken at the third visit. For each specimen the dentists scored the plaque extension according to a 100-mm visual analogue scale guided by two reference pictures showing examples of maximum (100%) and minimum plaque extension (0%) scored as 100 and 0 mm, respectively. For each specimen the mean score of the 5 dentists was calculated.

Saliva Analysis

Analyses of the saliva composition were performed on saliva collected on the second and third visits and independently of the micro-radiographic analyses. Concentrations of sodium, potassium, and

calcium (mM) were determined by atomic absorption spectroscopy, chloride (mM) by coulometric titration, total phosphate (mM) and protein ($\mu\text{g/ml}$) by colorimetric reactions, and amylase activity ($\mu\text{kat/l}$) by the PhadebasTM test kit. The degree of saturation of saliva with respect to hydroxyapatite as well as the critical pH (i.e. the pH where the ion product for hydroxyapatite is equal to the solubility product of hydroxyapatite) was calculated as previously described [Bardow et al., 2001].

Protein Profiles

Characterization of the protein profile from each subject was done by adding 2.5 μg of unstimulated saliva protein to SDS polyacrylamide gels with a gradient from 5 to 20% and afterwards fixed at 9%. The gels were silver stained and an alternative fixative containing 0.05% glutaraldehyde, 80% saturated picric acid, and 20% ethanol was used [Kirkeby et al., 1993]. Salivary amylase was identified by its activity on Western blots of the gels by exposing the blots to a humidified paste of the PhadebasTM amylase activity test kit for 24 h at 36 °C. Other salivary proteins were identified according their relative mobility in the silver stained gel [Schwartz et al., 1995] in relation to high and low molecular weight standards (Bio-RadTM). Quantification of salivary amylase was done by high-performance liquid chromatography (HPLC, integrated as the area under the curve of distinct peaks on the chromatogram) using an ion exchange column at pH 7.0. To verify which peaks represented amylase in the chromatogram the amylase activity was determined (Phadebas) in fractions for each peak obtained.

Experimental Caries

Root surface specimens were prepared from the mesial and/or distal root surfaces of surgically removed, unerupted human lower third molars. Prior to being mounted in the dentures, the specimens were cleaned mechanically and sterilized by exposure to ethylene oxide gas at temperatures not exceeding 55 °C while being kept wet at all times. The microradiographic analysis was performed on 200- μm -thick planoparallel sections cut at a distance of 1 mm from the border of the composite frame surrounding the specimens as previously described [Bardow et al., 2003]. The mineral profile was determined by means of the computer program Transverse-Microradiography (Inspector SystemsTM) and the following parameters were calculated: total mineral loss (vol% μm), lesion depth (μm) and mineral content in the surface layer (% sound dentin). An experienced technician who was blinded with regard to the study served as the final judge of each analysis.

Statistical Analysis

Excel and the free statistical software R version 1.3.1 (www.r-project.org) were used for analyses. To determine possible interrelations between variables, Spearman's rank correlation was used and the correlation coefficient (r_s) given. We preferred a non-parametric analysis due to the relatively small sample size, the presence of categorized variables, and the presence of many non-linear correlations. Major outcomes in terms of significance were subjected to the Bonferroni correction. To further illustrate the non-linear relationship between mineral loss and salivary composition variables non-linear curve fits (power law) were added to figures. Non-linear fits clearly had higher explanatory power (mean R^2 0.42) than alternative linear fits (mean R^2 0.30) for the two figures shown. The effect of possible confounders on key variables was analysed by multiple regression analysis. Intra-individual day-to-day variations between measure-

ments (i.e. days 0, 15, 45, and 62) were determined as the relative difference (with positive signs) in percent from the mean of the measurements. The level of significance was set at $p < 0.05$.

Results

Salivary Flow Rates

The mean unstimulated saliva flow rate (i.e. mean of the three measurements on days 0, 14, and 45) was 0.30 ± 0.05 ml/min (range 0.22–0.40 ml/min) with an intra-individual day-to-day variation of $16 \pm 13\%$. We did not define any inclusion criteria for the stimulated saliva flow rate, which therefore ranged considerably, from 0.60 to 2.22 ml/min with a mean of 1.42 ± 0.47 ml/min and an average intra-individual day-to-day variation of $13 \pm 12\%$.

Demineralization Parameters

Microradiographic analyses showed that all subjects developed carious lesions within the experimental period of 62 days, although at varying degrees. Mean mineral loss was $7,767 \pm 6,316$ vol% μm , mean lesion depth was 320 ± 161 μm , and the mean mineral content of the surface layer was $51 \pm 19\%$ (of sound dentine). Except for 2 subjects who had experienced high mineral losses the remaining 18 subjects were relatively evenly distributed in the range from 2,000 to 13,000 vol% μm (fig. 1).

Identifying Determinants for the Demineralization Parameters

No significant correlations were found between medication intake, feeling of dry mouth, sugar intake, and the demineralization parameters. The plaque index, gingival index, calculus index, and total DMFS did not show any significant correlation with the demineralization parameters either. However, the presence of active enamel caries showed significant correlations with the mineral loss ($r_s = 0.46$; $p < 0.05$) and mineral content in the surface layer ($r_s = -0.52$; $p < 0.05$) of the specimens. The mineral loss was higher and the mineral content in the surface layer was lower in the presence of active enamel caries. No significant correlations were obtained with the *Lactobacillus* level in saliva, plaque extension on the specimens, and the demineralization parameters. But there was a tendency for the mineral loss to be higher in the presence of high numbers of lactobacilli in saliva and high plaque extension scores on the specimens (r_s 0.36 and 0.40, respectively). Furthermore, the salivary *Lactobacillus* level seemed to become lower in the presence of high concentrations of

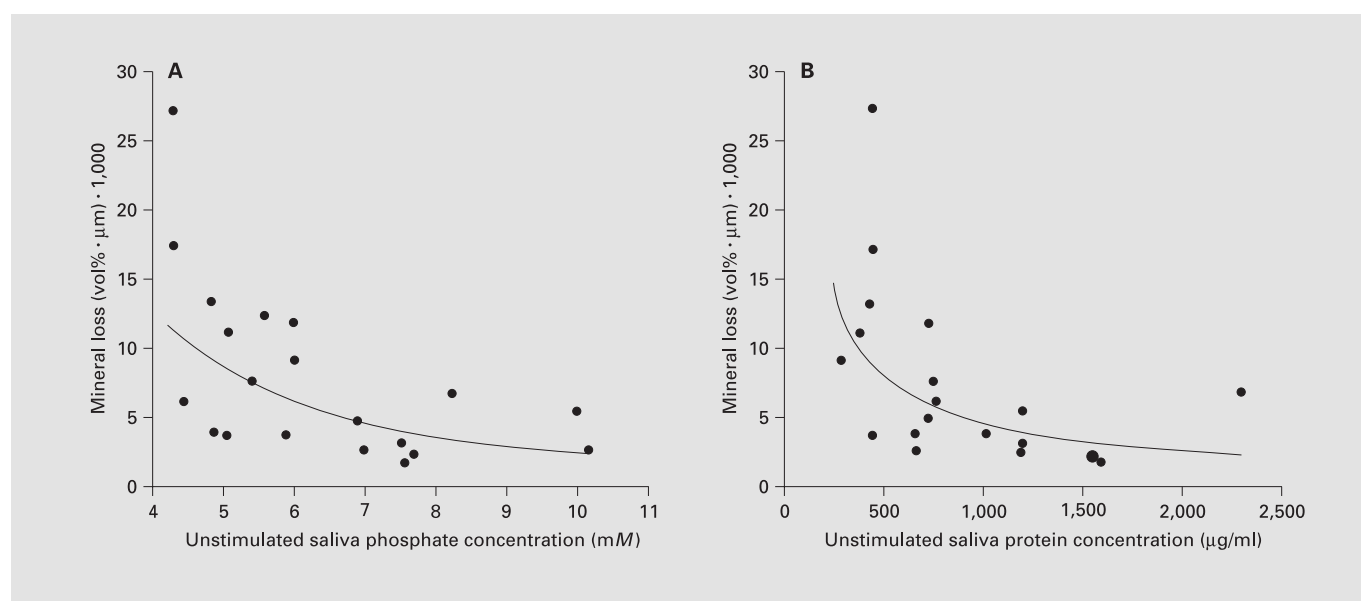


Fig. 1. Effects of unstimulated saliva total phosphate (**A**) and total protein (**B**) concentrations on mineral loss.

Table 1. Correlation matrix showing significant correlations obtained between saliva and demineralization parameters

	UWSPHOS	UWSPROT	UWSCPH	β-UWS	β-SWS	UWS
UWSPHOS	1.00					
UWSPROT	0.60 ^b	1.00				
UWSCPH	-0.55 ^c	-0.40	1.00			
β-UWS	0.77 ^a	0.43	-0.68 ^b	1.00		
β-SWS	0.50 ^c	0.44	-0.35	0.50 ^c	1.00	
UWS	-0.27	-0.36	0.50 ^c	-0.33	-0.15	1.00
ΔZ	-0.72 ^a	-0.70 ^b	0.51 ^c	-0.45 ^c	-0.48 ^c	0.28
LD	-0.54 ^c	-0.58 ^c	0.45	-0.43	-0.44	0.15
MCS	0.59 ^c	0.59 ^c	-0.54 ^c	0.47 ^c	0.55 ^c	-0.24

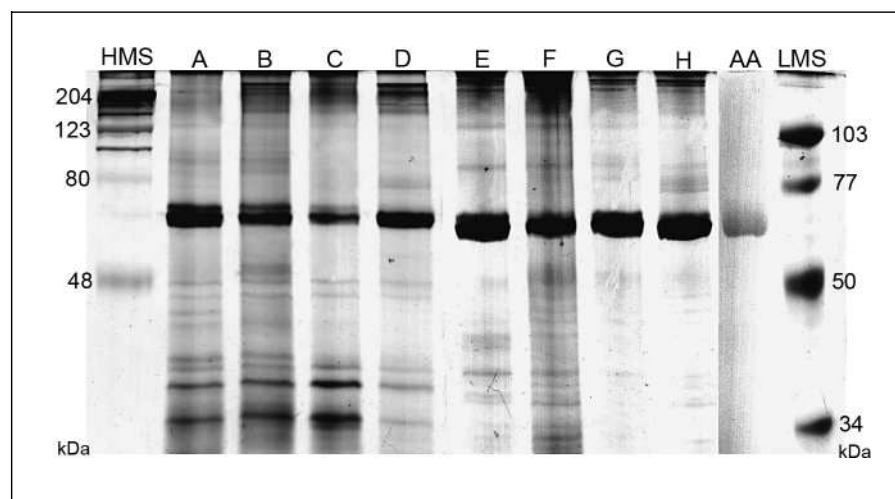
UWSPHOS, UWSPROT, UWSCPH, β-UWS, β-SWS, and UWS denote the unstimulated whole saliva phosphate and protein concentrations, critical pH with regard to hydroxyapatite, unstimulated and stimulated whole saliva buffer capacities, and flow rate, ΔZ mineral loss, LD lesion depth, and MCS mineral content of the surface layer. Correlation coefficients (r_s) were obtained by Spearman's rank correlation analysis; ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$. No significant correlations were obtained with saliva pH and bicarbonate concentration.

total protein in unstimulated saliva ($r_s = -0.47$; $p < 0.06$). Neither the unstimulated nor the stimulated saliva flow rates showed any significant correlations with the demineralization parameters. However, several significant correlations were obtained between the salivary compositional variables and the demineralization parameters.

Determinants of Demineralization

As shown in table 1 the most significant correlations with demineralization parameters were obtained with the unstimulated saliva total phosphate and protein concentrations, which in this study were independent of saliva flow rate. High concentrations of total phosphate and pro-

Fig. 2. Individual unstimulated saliva protein profiles were determined on silver-stained 9% SDS gels. The figure shows a selection of 8 subjects; lanes A–D show 4 subjects who developed the highest mineral loss in this study and lanes E–H show 4 subjects who developed the lowest mineral loss. HMS = High-molecular weight standard; LMS = low-molecular weight standard; AA = amylase activity obtained on Western blots exposed to the amylase activity test kit. The saliva alpha-amylase band (57–65 kDa) is broader and more intensely stained in lanes E–H compared to lanes A–D, indicating a higher amylase concentration and activity in subjects E–H compared to A–D.



tein in unstimulated saliva resulted in low mineral loss and lesion depth. The unstimulated saliva total phosphate and protein concentrations also had a significant impact on the mineral content in the surface layer that became less demineralized with high salivary concentrations of these variables. Bonferroni corrected *p* values were 0.028 for the total phosphate mineral loss correlation and 0.044 for the total protein mineral loss correlation. These values reveal that the correlations were significant also when the relatively high number of correlations tested between mineral loss and saliva variables, which was 28 correlations in total, was taken into consideration.

Several other correlations, although less significant, were obtained between demineralization parameters and the unstimulated saliva critical pH, unstimulated saliva buffer capacity (in the acidic pH range from pH 3 to 5), and stimulated saliva buffer capacity (in the acidic pH range from pH 3 to 5). However, these variables were generally more correlated with the unstimulated saliva phosphate concentration than with the demineralization parameters as shown in the upper part of the matrix. Thus, the major determining variables for the demineralization parameters were the unstimulated saliva total phosphate and protein concentrations, which were also relatively constant variables throughout the study period with day-to-day variations of 12 ± 12 and $21 \pm 17\%$, respectively.

Figure 1A, B shows the significant effect of unstimulated saliva phosphate (adjusted R^2 0.45; $p < 0.01$) and protein concentrations (adjusted R^2 0.37; $p < 0.05$) on mineral loss. The explanatory power of a model containing unstimulated phosphate concentration against mineral

loss could not be increased by adding other variables to the model, nor could the phosphate and protein concentrations coexist in the same model due to their mutual relation (table 1). However, the explanatory power of a model containing logarithmically transformed unstimulated saliva protein concentration against mineral loss (estimate $-22,669 \pm 4,500$ vol% μm ; $p < 0.001$) was increased when sugar intake (ordinary sugar and sweet fruit) (estimate $1,432 \pm 421$ vol% μm ; $p < 0.01$) and the ratio of filled/non-filled tooth surfaces (estimate $2,828 \pm 950$ vol% μm ; $p < 0.05$) were added to the model (adjusted R^2 0.75; $p < 0.001$).

Qualitative Protein Analysis

Since the total protein concentration in unstimulated saliva exhibited a high explanatory power with respect to mineral loss, all individual protein profiles were determined. Electrophoretic separation of the proteins showed a distinct band with a molecular weight between 57 and 65 kDa, identified mainly as the salivary alpha-amylase band by its activity and mobility, to become broader and more intensely stained in individuals with low mineral loss (fig. 2). In agreement with electrophoretic findings a significant negative correlation was obtained with the unstimulated saliva amylase activity (in suspension) and the mineral content in the surface layer ($r_s = -0.52$; $p < 0.05$). Furthermore quantification of salivary amylase by HPLC showed three distinct peaks in the chromatograms, identified as amylases by their amylase activity, to have a significant negative correlation with mineral loss ($r_s = -0.55$; $p < 0.05$). However, 65% of the variance in amylase activity and 39% of the variance in HPLC measure-

ments could be explained by the total protein concentration in unstimulated saliva ($p < 0.001$). Thus high total protein concentrations were indicative of high amylase activities and concentrations.

Discussion

This study has shown that saliva composition has an effect on demineralization parameters such as mineral loss, lesion depth and mineral content of the surface layer in experimental root caries lesions *in situ*. The unstimulated saliva composition was considerably more important for this relation than the stimulated saliva composition. This finding is in accordance with previous calculations regarding the effect of saliva on oral clearance, which is also mostly determined by unstimulated saliva [Dawes, 1983]. We therefore speculate that the effect of saliva on caries lesion development in general is mostly attributed to its unstimulated flow and composition.

The most significant correlations with all demineralization parameters were obtained with the unstimulated saliva total phosphate and protein concentrations. High salivary concentrations of these constituents were protective against caries lesion development. As the day-to-day variation for these salivary variables was relatively low we assume that the individual exposure to these salivary constituents had been relatively constant throughout the experimental period. However, it is difficult to pinpoint the underlying mechanisms responsible for the correlations obtained. Phosphate can act as a buffer [Lilienthal, 1955; Bardow et al., 2000b], increase the degree of saturation, and decrease the critical pH with respect to hydroxyapatite [Ericsson and Oberg, 1952]. Proteins can also function as buffers [Lilienthal, 1955; Bardow et al., 2000b] as well as have other functions such as forming the acquired pellicle [for a review, see Lendenmann et al., 2000] and having antimicrobial properties [for reviews, see Lenander-Lumikari and Loimaranta, 2000; Amerongen and Veerman, 2002].

That some of these specific functions have an effect is further supported by the finding that a low critical pH and a high buffer capacity in the acidic pH range were protective against caries lesion development (table 1). In this study 91% of the variance in critical pH could be explained by the saliva calcium and phosphate concentrations ($p < 0.001$) and up to 47% of the variance in saliva buffer capacity could be explained by the saliva phosphate and protein concentrations ($p < 0.05$). It could therefore be that high saliva phosphate and protein con-

centrations, which lead to low critical pH and high saliva buffer capacity, may have the potential to reduce the demineralization of actively progressing carious lesions like the ones developed in this study. The results of the qualitative and quantitative saliva protein analyses revealed correlations between the unstimulated saliva amylase and caries lesion development. These findings are in agreement with some of the few other studies on this topic [Anderson and Mandel, 1982; Banderas-Tarabay et al., 2002] showing an effect of salivary protein composition on carious lesion development. Therefore, the results also point towards specific salivary proteins having an effect on carious lesion development, including an effect of salivary amylase [for a review, see Aguirre et al., 1993]. However, phosphate and proteins diffuse through plaque differently, as small molecules diffuse much faster through plaque than large molecules [Thurnheer et al., 2003]. Therefore the areas in the plaque where these compounds had an effect might have been quite different. Thus some antimicrobial effects originating from large molecules may have been limited to the most superficial part of the plaque.

Although the saliva composition showed an effect on demineralization parameters in this study it is important to note that the study group and experimental conditions were homogeneous and stratified with regard to unstimulated saliva flow rate, fluoride exposure, oral hygiene level, and time of lesion development. Furthermore, the longitudinal design of this study implied that the saliva analyses were carried out during caries lesion development and not after the lesions had developed as in cross-sectional studies. However, the fact that past caries experience and active enamel caries in the natural dentition showed relations to experimental lesion development suggests that the correlations obtained in this study might be of general significance. Furthermore these findings also support the general belief that past caries experience is a good predictor for future caries [Hausen, 1997].

In conclusion, this study has shown that the composition of unstimulated saliva can explain a substantial part of experimental caries lesion development; especially phosphate, protein, and amylase concentrations in unstimulated saliva may be of importance.

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